Secondary Mitochondrial Respiratory Chain Defect Can Delay Accurate PFIC2 Diagnosis

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Abstract Multiple respiratory chain deficiencies represent a common cause of mitochondrial diseases and often result in hepatic failure. There is no gold-standard test for diagnosing mitochondrial disease, and the current diagnosis relies on establishing a consistent pattern of evidence from clinical data, neuroimaging, tissue biopsy, and biochemical investigations. In some patients, the mitochondrial respiratory chain defect (MRCD) diagnosis is confirmed by genetic investigations. In most cases, genetic investigations

are not informative and a number of cases remain unexplained.

Here, we report on two children presenting with liver disease in whom first investigations suggested MRCD, due to decreased liver respiratory chain activities and decreased mitochondrial DNA copy number. However, sequencing of the genes known to be associated with mitochondrial DNA instability did not identify any pathogenic mutations. Further investigations including exome analysis, biliary bile salt analysis, and/or BSEP immunostaining detected a defect in the bile salt export pump (BSEP). Diagnosis of progressive familial intrahepatic cholestasis type 2 (PFIC2), a hereditary disorder in bile formation due to BSEP deficiency was confirmed by *ABCB11* gene sequencing. Deleterious mutations were identified in both patients: one harboring compound heterozygous mutations (p.Arg470*/c.1308+2T>A) and the other homozygous nonsense mutation (p.Tyr354*). This report increases awareness of a possible secondary mitochondrial respiratory chain defect in the liver tissue associated with other underlying causes such as PFIC2.

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List of Abbreviations

BSEP	Bile salt export pump
DGUOK	Deoxyguanosine kinase
GGT	Gamma-glutamyl transferase
MRCD	Mitochondrial respiratory chain defect
mt	Mitochondrial
PFIC2	Progressive familial intrahepatic cholestasis type 2
POLG	Polymerase gamma
UDCA	Ursodeoxycholic acid

Introduction

Mitochondrial respiratory chain deficiencies may present as liver disease with cholestasis and/or liver failure within the first months of life (Molleston et al. 2013). There is no gold-standard test for mitochondrial respiratory chain defect (MRCD) diagnosis, which usually relies on establishing a pattern of evidence from clinical, biochemical, pathological, and radiological data (Cormier-Daire et al. 1997; Rustin et al. 1994). The primary origin of MRCD is likely when a specific genetic defect is identified, a situation that rarely occurs due to the huge number of candidate genes (Rustin et al. 1994; Gaignard et al. 2013; Molleston et al. 2013). Recently, Molleston et al. (2013) established a tiered approach to guide the evaluation of suspected mitochondrial disease. Clinical presentation of MRCD is heterogeneous and several organs may be affected including liver, brain, muscle, and/or kidney (Cormier-Daire et al. 1997; Morris 1999). Isolated liver disease, observed in some cases, may represent an indication to liver transplantation, whereas multiorgan injury represents a contraindication (Dubern et al. 2001). We report here on two children in whom liver disease initially thought to be akin to MRCD was due to progressive familial intrahepatic cholestasis type 2 (PFIC2).

Case Reports

Patient 1 was born at term from healthy non-consanguineous parents and experienced physiological neonatal jaundice without discolored stools. She was referred to Bicêtre hospital at age 6 months for cholestasis, pruritus, hepatosplenomegaly, and growth delay since age 4 months. Serum liver tests showed increased bilirubin and bile salt levels, elevated transaminase activities, and slight increase of gamma-glutamyl transpeptidase (GGT) activity. Evidence for mild liver failure and increased alpha-fetoprotein level were observed. Normal lactate levels were found in blood and cerebrospinal fluid (Table 1). Liver ultrasonography showed signs of cirrhosis with portal hypertension and nodules and normal biliary tract. Liver histology revealed hepatocellular cholestasis, micronodular cirrhosis associated with giant hepatocytes. Cytoplasmic aspect of hepatocytes was eosinophilic (oncocytic) as seen in MRCD (Fig. 1), but no steatosis was observed. Neurological examination, brain MRI, and heart ultrasonography were normal. MRCD was suspected due to low respiratory chain complex levels and reduced copy number of mitochondrial DNA (mtDNA) in the liver (Table 2) and liver histology. Genetic investigations, including mtDNA analysis by long range PCR, *MPV17*, *TRMU*, deoxyguanosine kinase (*DGUOK*), and polymerase gamma (*POLG*) gene sequencing, failed to find any mutation.

Liver function worsened, ursodeoxycholic acid (UDCA) therapy failed, and the child underwent a liver transplantation at 2 years for MRCD isolated to the liver. Bile was collected at this time and biliary lipid analysis revealed bile salt secretion deficiency (biliary bile salt 0.17 mmol/L, N>10), a sign suggestive of PFIC2. Immunostaining on explanted liver showed absence of BSEP (bile salt export pump) at the canalicular membrane, while canalicular MDR3 expression was normal (Fig. 1). All coding exons of *ABCB11* were amplified together with their respective exon/intron boundaries and sequenced. This analysis identified biallelic heterozygous mutations: a nonsense variation (c.1408 C>T; p.Arg470*) previously reported (Davitt-Spraul et al. 2010) and a novel splice variation of exon 12 (c.1308+2T>A). In silico analysis of this latter variation predicted exon skipping due to disappearance of the donor splice site.

Patient 2 was born at term from healthy consanguineous parents and had intrauterine growth retardation. She presented with cholestatic jaundice from birth. She was referred to Bicêtre hospital at age 3 months for investigation of cholestasis. She had hepatosplenomegaly and growth delay. Liver tests showed hyperbilirubinemia, elevated serum transaminase activities, and slightly elevated serum GGT activity (Table 1). At 5 months of age, she had liver failure and development delay with axial and peripheral hypotonia. Blood tests showed severe lactic acidosis and increased alpha-fetoprotein level. She had severe hypoglycemia (Table 1), which remained unexplained despite appropriate investigations (data not shown). Heart ultrasound examination and brain MRI were normal. Ultrasonography showed an enlarged homogeneous liver. Liver histology showed giant hepatocytes, hepatocellular cholestasis, and panlobular fibrosis. No steatosis was observed. The neurological status led to the decision to contraindicate liver transplantation. UDCA therapy failed and the child died of liver failure at 7 months of age. Respiratory chain complexes showed low levels of complex I and III in liver biopsy and qPCR showed a reduced copy number of mtDNA (Table 2). MtDNA analysis by long range PCR excluded large deletion of mtDNA. At that time no other genetic investigation of MRCD was performed, but exome analysis was performed 10 years later in order to explain MRCD. This analysis revealed *ABCB11* defect, which was further confirmed using direct sequencing of *ABCB11* (homozygous mutation: c.1062T>A; p.Tyr354*).

In both children, other causes of cholestasis and/or liver failure known to present within the first year of life were excluded (Debray et al. 2001). Exon and exon-intron junctions of *POLG*, *DGUOK*, *MPV17*, *TRMU*, and *ABCB11* genes were sequenced as previously described as well as liver immunostaining analysis (Davitt-Spraul et al. 2010; Gaignard et al. 2013).

Table 1 Biochemical investigations in the two patients with PFIC2

Patient age at investigation	Patient 1 6 months	Patient 2 3 months	Patient 2 5 months
Blood tests*			
Total/conjugated bilirubin (<17 µmol/L)	417/276	223/143	359/247
Bile salts (<15 µmol/L)	310	nd	nd
GGT (<45 IU/L)	69	49	59
Alanine aminotransferase (<40 IU/L)	634	2064	1041
Creatine phosphokinase (<260 IU/L)	70	nd	46
Alpha-fetoprotein ^a (<7 IU/mL)	117000	nd	13400
Prothrombin time ^b (>70 %)	55	100	49
Factor V (>80 %)	61	100	50
Albumin (>35 g/L)	39	nd	28
Fasting glucose (3.2–5.5 mmol/L)	3.8	nd	0.9
Lactate (0.5–1.7 mmol/L)	1.6	nd	8.0
Pyruvate (<0.15 mmol/L)	0.111	nd	0.300
Lactate/pyruvate ratio (8–18)	14	nd	27
Ammonium (<50 µmol/L)	58	nd	69
Cerebrospinal fluid analysis*			
Lactate (0.6–1.9 mmol/L)	1.3	nd	3.8
Pyruvate (<0.15 mmol/L)	nd	nd	0.18
Lactate/pyruvate ratio (8–18)	nd	nd	21

nd not determined

*Normal values are given in parenthesis

^a Values after intravenous injection of vitamin K. GGT, gamma-glutamyl transferase; normal GGT range for age: 1 month (10 to 270 IU/L), 2 months (10 to 160 IU/L), 4 months (7 to 100 IU/L), >5 months (5 to 45 IU/L)

^b Normal alpha-fetoprotein range for age: 1 month (<5,600 IU/mL), 3 months (< 180 IU/mL), 5 months (<70 IU/mL), 6 months (<20 IU/mL), > 6 months (<7 IU/mL)

Discussion

In most circumstances, the diagnosis of MRCD affecting the liver is based on the analysis of mitochondrial respiratory chain complex activities measured on liver tissue (Cormier-Daire et al. 1997; Rustin et al. 1994). Experimentally it has been shown that cholestasis and cirrhosis may affect the function of mitochondrial respiratory chain complexes (Krähenbühl et al. 1994, 1995). Therefore, in case of cholestasis and/or liver failure, there is a risk to overdiagnose liver MRCD, the decrease in mitochondrial respiratory chain complex activities and/or in mtDNA copy number being secondary to liver injury. The data reported here in two children show that secondary MRCD may be due to liver disease such as PFIC2.

Since cases of MRCD affecting the liver have been previously described, it was difficult to not consider MRCD in the two patients reported here, all the more that both had additional signs that could evoke MRCD (Cormier-Daire et al. 1997; Dubern et al. 2001). These findings described below, together with the decrease of liver mitochondrial respiratory chain complex activity and of mtDNA copy number, have led to a wrong diagnosis of MRCD. Patient 1 had an oncocytic aspect of hepatocytes, a finding not reported so far in PFIC2 and described in MRCD

(Morris 1999; Davit-Spraul et al. 2010). Analysis of biliary bile acids performed at the time of liver transplantation has suggested PFIC2, biliary bile acid concentration being <1 mmol/L (Davit-Spraul et al. 2010). This diagnosis was confirmed by BSEP immunostaining and *ABCB11* sequencing. Patient 2 had intrauterine growth retardation and development delay with hypotonia, hypoglycemia, and elevated blood and cerebrospinal fluid lactate/pyruvate ratios – findings not reported so far in PFIC2 and described in MRCD (Cormier-Daire et al. 1997; Dubern et al. 2001; Davit-Spraul et al. 2010). Exome analysis performed to elucidate the genetic basis of MRCD established PFIC2 diagnosis. It is very likely that in both children, signs evocative of MRCD were due to a secondary dysfunction of mitochondrial respiratory chain induced by primary liver injury of PFIC2. Indeed, both patients presented with a moderate reduction of mtDNA copy number (20–30%), which retrospectively is likely due to a secondary MRCD rather than a primary genetic defect. The presence of mtDNA depletion syndrome with liver mtDNA copy number below 10% is highly suggestive of a primary genetic defect (Molleston et al. 2013; Sarzi et al. 2007). Nevertheless, in patients exhibiting mtDNA depletion syndrome with a less pronounced decrease in liver mtDNA copy number (10–40 %), a primary defect cannot be

Fig. 1 Liver histology and liver immunostaining studies in patient 1. **(a)** Patient: Picro Sirius staining, x40. Presence of hepatocellular cholestasis with a canalicular bile plug (*arrow*) and of lobular fibrosis. **(b)** Patient: Hematoxylin and eosin (HES), x40. Note the oncocytic aspect of hepatocytes (*arrow*). Presence of multinucleated and giant hepatocytes

(*arrowheads*, also in panel C). **(c)** Patient: BSEP immunostaining, x40. Absence of BSEP canalicular expression. **(d) Upper panel:** Patient, MDR3 immunostaining, x40. MDR3 is expressed at the canalicular membrane (*arrow*). **Lower panel:** Normal human liver, BSEP immunostaining, x40. BSEP is expressed at the canalicular membrane (*arrow*)

Table 2 Enzyme activities of mitochondrial respiratory chain complexes and molecular analysis of mitochondrial DNA in the liver of the two patients with PFIC2

	Respiratory chain activities in liver biopsy ^a		
	P1	P2	Control values
<i>Complex activities</i> (nmol/min/mg proteins)			
Complex I	5	5	19–26
Complex II	83	75	168–277
Complex III	73	114	143–192
Complex I + III	29	11	41–84
Complex II + III	31	40	66–103
Complex IV	102	216	202–319
Citrate synthase	65	58	63–131
Complex IV/CS	1.6	3.7	2.5–3.3
Complex IV/II	1.2	2.9	0.9–2.1
Complex IV/III	1.4	1.9	1.0–1.8
Complex IV/I	20.4	43.2	6.0–10.0
<i>Molecular analysis</i>			
Mt DNA long range PCR	Normal	Normal	Normal
Mt DNA copy number	27%	30%	100%

^a Surgical liver biopsies were performed in patients 1 and 2, at 6 and 5 months of age, respectively, when patients had liver failure (Table 1). *Mt DNA* mitochondrial DNA, *CS* citrate synthase. Mitochondrial enzymatic activities of respiratory chain complexes (I, II, III, and IV) and mitochondrial enzyme marker (citrate synthase) were measured in liver biopsies (2). Mt DNA was analyzed by long range PCR and mt DNA copy number was measured in liver biopsies by quantitative PCR based on the ratio of mt DNA to nuclear DNA (*MTND2/ATP5B*), according to Chabi et al. 2003

excluded. In such situation, a secondary defect can also be observed (Helbling et al. 2013). Previous studies reported bile acid toxicity on oxidative mitochondrial metabolism affecting complex II, III, and IV activities (Krähenbühl et al. 1994, 1995). Thus, cholestasis could be responsible for and explained the reduced mitochondrial respiratory chain complex activities observed in our patients. Also, the reduced mtDNA copy number seen in our patients might be due, at least in part, to liver failure as it has also been shown in other patients undergoing liver transplantation for acute liver disease (Helbling et al. 2013).

The data presented here show that in presence of signs of MRCD in a child with cholestasis and liver failure, a PFIC2 should be considered. This, all the more that routine blood biological markers (increased alanine aminotransferase > fivefold normal, increased alpha-fetoprotein for age, normal or slightly elevated GGT, increased bile acids), pruritus, liver histology (giant hepatocytes, hepatocellular cholestasis), may suggest PFIC2 and that the proof for MRCD remains uncertain (Davit-Spraul et al. 2010). Otherwise, there is a risk to overdiagnose MRCD and to wrongly contraindicate liver transplantation in case of extrahepatic involvement, such as a neurological injury, potentially reversible and not due to primary MRCD. Another risk is to overdiagnose PFIC2 in case of MRCD isolated to the liver. This risk has minimal consequences since a good outcome is expected after liver transplantation, while the appearance of a delayed neurological involvement cannot be formally excluded (Dubern et al. 2001). Currently, in such situation, BSEP liver immunostaining and *ABCB11* sequencing should be performed first and may help to differentiate between both entities.

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One-Sentence Take-Home Message

Hepatic mitochondrial respiratory chain defect may be secondary to severe cholestasis and liver failure.

Contribution of Individual Authors

Anne SPRAUL: identification of patient defect (genetic analysis), manuscript writing

Marine BEINAT: identification of patient defect (exome analysis)

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Emmanuel JACQUEMIN: diagnosis and follow-up of patient 1, manuscript writing and supervision

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